

Cultured human keratinocytes cannot metabolize vitamin D₃ to 25-hydroxyvitamin D₃

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The metabolism of [³H]vitamin D₃ was studied in cultured human keratinocytes (CHK). Intact CHK were incubated for 1, 6, 12, 24 and 48 h with [³H]vitamin D₃ and the lipid soluble fractions from the media and cells were extracted by high-performance liquid chromatography (HPLC). Vitamin D₃ and its metabolites, 25-OH-D₃, 24,25(OH)₂D₃ and 1,25(OH)₂D₃ were added to the extracts, as markers, prior to HPLC. HPLC analysis of the lipid extracts did not reveal any monohydroxylated metabolites. CHK incubated for one hour with [³H]25-OH-D₃ showed a 10±4% conversion to [³H]1,25(OH)₂D₃, whereas no conversion to [³H]1,25(OH)₂D₃ was observed in control CHKs that were boiled prior to incubation with [³H]25-OH-D₃. These findings suggest that cultured neonatal keratinocytes are incapable of metabolizing vitamin D₃ to 25-OH-D₃.

Cultured human keratinocyte; Vitamin D₃; 25-Hydroxyvitamin D₃

1. INTRODUCTION

When human skin is exposed to sunlight, vitamin D₃ is produced [1]. Once formed, vitamin D₃ is transported via a vitamin D-binding protein to the liver where it is metabolized to 25-OH-D₃, which is further metabolized in the kidney to 1,25(OH)₂D₃, the most biologically active form of vitamin D₃ [2].

Receptors for 1,25(OH)₂D₃ have been demonstrated in cultured human and mouse keratinocytes [3,4] and in cultured human dermal fibroblasts (CHDF) [3,5,6]. Incubation of both cultured human keratinocytes (CHK) and CHDF with 1,25(OH)₂D₃ caused a dose-dependent decrease in cell proliferation and in CHK, an increase in differentiation [7].

Cultured neonatal keratinocytes metabolize 25-OH-D₃ to 1,25(OH)₂D₃ [8]. Since the skin is the site for vitamin D₃ synthesis and a target organ for its 1,25-dihydroxy metabolite, it was of interest to determine whether CHK could also metabolize vitamin D₃ to 25-OH-D₃.

2. METHODS AND MATERIALS

2.1. Cultured human keratinocytes

Cultured human keratinocytes were grown in serum-free conditions as previously described [7]. Briefly, the medium consisted of Dulbecco's modified Eagle's medium (DMEM) with 0.15 mM calcium (M.A. Bioproducts, Walkersville, MD, USA) containing 7 growth fac-

tors: transferrin (5 µg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (203 ng/ml), insulin (5 µg/ml), prostaglandin E-1 (50 ng/ml), cholera toxin (0.1 µg/ml, Sigma, St. Louis, MO, USA), and selenous acid (2 ng/ml, Collaborative Research, Lexington, MA, USA). Penicillin G (75 U/ml) and streptomycin (50 ng/ml) were also added to the cultures. Keratinocytes were isolated from human neonatal foreskin according to a modification of the method of Rheinwald and Green [9]. For the experiments studied, fresh medium containing vehicle alone (0.01% ethanol) or one of the following compounds 1,25(OH)₂D₃, 24,25(OH)₂D₃ (gifts from Dr. M. Uskokovic of Hoffmann-La Roche, Nutley, New Jersey), 25-OH-D₃ (a gift from J. Babcock of Upjohn, Kalamazoo, Michigan) or vitamin D₃ (Sigma, St. Louis, MO) was added to each culture. Radioactive vitamin D₃ was from Amersham, TRK 346, Batch 33, [1α,2α-³H]vitamin-D₃, 18.3 Ci/mmol.

2.2. Incubation of cultured human keratinocytes with [³H]vitamin D₃

Triplicate 35 mm cultures of CHK that were approximately 80% confluent were incubated with 100 000 cpm of [³H]vitamin D₃ for 1, 6, 12, 24, or 48 h. The medium was collected and the cells washed 2 times with medium which was combined with the first. Lipid extracts were obtained separately from the cells and the medium by adding 7 ml of methanol and 5 ml of H₂O and then transferring the mixture to a separating flask. Methylene chloride (4 ml) was added to the flask until a precipitate formed, and then an additional 16 ml was added. After mixing, the lipid-soluble (bottom) layer was removed and dried with nitrogen. To this, 20% IPA/hexane was added and the mixture was dried down with nitrogen. Cold standards were added to the extract (16 ng/ml of 1,25(OH)₂D₃, 35 ng/ml of 25-OH-D₃, 1 ng/ml of vitamin D₃ and 20 ng/ml 24,25(OH)₂D₃) prior to analysis by HPLC. Samples were eluted at a flow rate of 2.5 ml/min with a step-gradient of isopropanol in *n*-hexane on a 25×4.5 mm Zorbax-sil column as follows; 1.25% from 0 to 15 min; 2.5% from 16 to 35 min, and 5% from 36 to 70 min. The various standards were monitored by their UV absorbance at 254 nm. One ml fractions were collected and counted in a liquid scintillation counter. This system was used to separate the vitamin D₃, 25-OH-D₃, 24,25(OH)₂D₃ and 1,25(OH)₂D₃. For the study of the conversion of 25-OH-D₃ to 1,25(OH)₂D₃, the step-gradient of isopropanol in *n*-hexane was changed to 2.5% for 0 to 40 min and 5% for 41 to 70 min and the flow rate was 2.0 ml/min.

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3. RESULTS

To be certain that [^3H]vitamin D_3 entered the keratinocytes, the cells were incubated with [^3H]vitamin D_3 and then at various times the cells and medium were harvested. At 1, 12 and 24 h after its addition, $28 \pm 5\%$, $50 \pm 9\%$ and $61 \pm 6\%$, respectively, of the [^3H]vitamin D_3 entered the cells and was available to intracellular enzymes. Accumulation of [^3H]vitamin D_3 peaked at 24 h with no significant difference from 24 ($61 \pm 6\%$) to 48 h ($66 \pm 8\%$), $P > 0.05$. At all times tested, $88 \pm 6\%$ of the radioactivity was recovered from both the cells and the medium as [^3H]vitamin D_3 . The other $12 \pm 4\%$ was recovered in the water soluble phase of the extraction. A comparison of HPLC chromatograms of the standards (Fig. 1A) with the lipid extracts of cells (Fig.

1B,C) and medium (Fig. 1D,E) following incubation with [^3H]vitamin D_3 for 1 h (Fig. 1B,D) and 12 h (Fig. 1C,E) demonstrated that there was no metabolism of vitamin D_3 to known mono- and di-hydroxylated metabolites. Similar results were obtained from both cells and medium for the 15 min, 6 and 48 h incubations (data not shown). To be certain that the CHK retained the capacity to metabolize 25-OH-D_3 , CHK were incubated, in triplicate, with [^3H]25-OH- D_3 for 1 h. As seen in Fig. 2A, 10% of the [^3H]25-OH- D_3 converted to [^3H]1,25(OH) $_2\text{D}_3$. The overall conversion for the triplicate samples was $10 \pm 4\%$. The [^3H]1,25(OH) $_2\text{D}_3$ peak was collected and shown to migrate with 1,25(OH) $_2\text{D}_3$ standard (data not shown). CHKs that were boiled prior to incubation with [^3H]25-OH- D_3 , demonstrated no conversion to [^3H]1,25(OH) $_2\text{D}_3$ (Fig. 2B).

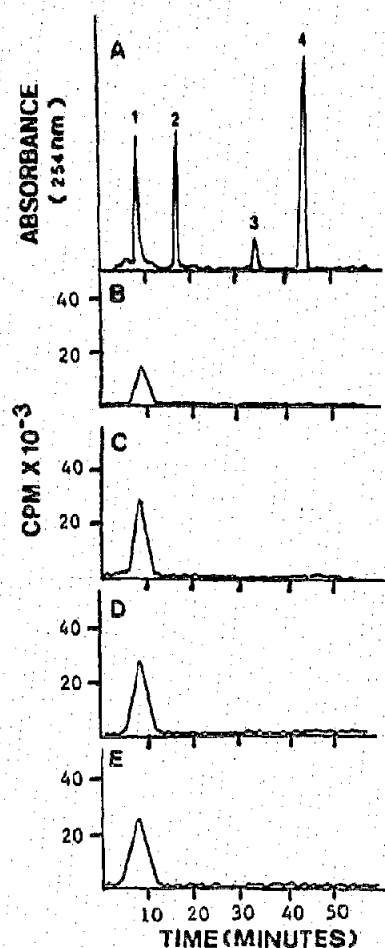


Fig. 1. Representative chromatograms from standards (A, 1, vitamin D_3 ; 2, 25-OH- D_3 ; 3, 24,25(OH) $_2\text{D}_3$; 4, 1,25(OH) $_2\text{D}_3$) and cultured human keratinocytes (B,C) and the incubation medium (D,E) after incubation with [^3H]vitamin D_3 (100 000 cpm) for 1 (B,D) and 12 h (C,E). The cells and the medium were kept separate and the lipid fractions were extracted and separated on HPLC (see section 2). 1 ml fractions were collected and counted on liquid scintillation counter.

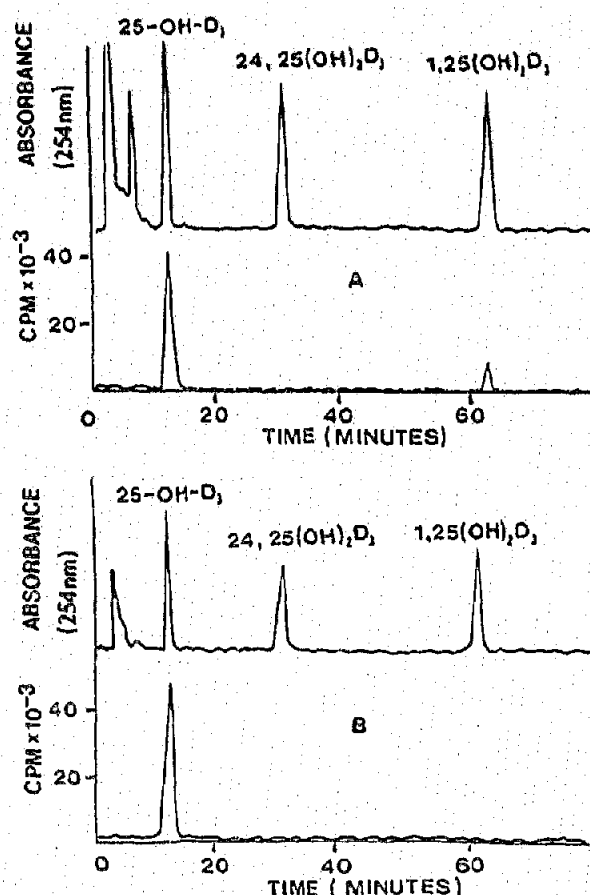


Fig. 2. Lipid extracts of cultured human keratinocytes (A) after 1 h incubation with [^3H]25-OH- D_3 at 37°C and analyzed by HPLC (see section 2). 1 ml fractions were collected and counted in a scintillation counter. The tritiated polar metabolite peak ($r_t = 61$ min) co-eluted with standard 1,25(OH) $_2\text{D}_3$. Fig. 2B is an HPLC chromatogram of the lipid extract of keratinocytes that have been boiled prior to incubation with [^3H]25-OH- D_3 . There was no conversion of the [^3H]25-OH- D_3 to [^3H]1,25(OH) $_2\text{D}_3$.

4. DISCUSSION

Investigators have shown that CHKs [3] possess nuclear receptors for $1,25(\text{OH})_2\text{D}_3$ and that human skin may be a target tissue for $1,25(\text{OH})_2\text{D}_3$ [6,7]. CHK metabolize 25-OH-D_3 to both $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ [7,8].

It has previously been shown [10] that hepatectomized, vitamin-D-deficient rats that received vitamin D_3 , maintained low, but detectable levels of 25-OH-D_3 . This suggests that the liver was not the sole site for the metabolism of vitamin D_3 to 25-OH-D_3 . Our results demonstrate that CHK, which convert 25-OH-D_3 to $1,25(\text{OH})_2\text{D}_3$, cannot metabolize vitamin D_3 to 25-OH-D_3 . These results suggest that the human epidermis is unable to metabolize sunlight induced vitamin D_3 to $1,25(\text{OH})_2\text{D}_3$.

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